Bioenergy: Challenges and Opportunities
International Conference and Exhibition on Bioenergy
April 6th – 9th 2008
Universidade do Minho, Guimarães, Portugal

ALCOHOLIC BEVERAGE FROM CHEESE WHEY: IDENTIFICATION OF VOLATILE COMPOUNDS

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ABSTRACT
A spirit was produced from cheese whey continuous fermentation by *Kluyveromyces marxianus*, and the volatile compounds present in this alcoholic drink were identified. Fermentation was performed in a 1000 L reactor at 30 ºC, with initial lactose concentration and hydraulic residence time of 50 g/L and 5 h (dilution rate 0.2 h^{-1}), respectively. The raw spirit (35.4% v/v ethanol) was obtained by distilling the fermentation broth in a pot still. Volatile compounds were quantified by gas chromatography, either by direct injection (with flame ionization detector) or after dichloromethane extraction (coupled with mass spectrometry). Higher alcohols were quantitatively the most abundant group of volatile compounds in this drink, with isoamyl alcohol, isobutanol, and 1-propanol being the most abundant (886.6 mg/L, 542.1 mg/L and 266 mg/L, respectively). Among the total esters, ethyl acetate showed the highest concentration (138.2 mg/L). Other components, such as the terpenes linalool, α-terpineol and geraniol were also identified. Considering that the quality of an alcoholic drink can be evaluated by the ratio between isoamyl alcohol/2-methyl-1-propanol and 2-methyl-1-propanol/1-propanol, which have to be higher than unity, it can be concluded that a novel spirit of acceptable organoleptic character could be produced by whey fermentation with *K. marxianus*.

Keywords: cheese whey; continuous fermentation; alcoholic beverage; volatile compounds; *Kluyveromyces marxianus*

INTRODUCTION
Whey is the liquid remaining after the precipitation and removal of milk casein during cheese-making. It represents about 85 to 95% of the milk volume [1] and its world production is estimated to be over 10^8 tons per year [2]. Biological treatment of whey by conventional aerobic process is very expensive and most milk plants do not have proper treatment systems for its disposal. As a consequence, around 47% of the produced amount is disposed in water bodies or loaded onto the land. However, whey disposal to the environment affects the physical and chemical structure of soil, decreasing crop yield; and the release into water bodies reduces aquatic life by depleting the dissolved oxygen [3]. Besides the environmental pollution aspect, dumping of whey constitutes a significant loss of potential food and energy, as it retains about 55% of total milk nutrients (lactose - 45 to 50 g/L; soluble proteins - 6 to 8 g/L; lipids - 4 to 5 g/L, mineral salts and others) [1, 3].
The production of an alcoholic beverage by bioconversion of whey is an alternative of great interest for reuse of this industrial by-product. Although numerous literature reports can be found about alcohol production from whey, most of them are based in the addition of fruit juices in whey. In addition, the scale-up of the process has been few explored and information regarding the volatile compounds presents in the distilled drink is scarce, since most of the studies are only concerned in increasing the ethanol yield during fermentation. Based on these facts, the goal of the present study was to produce an alcoholic drink by cheese whey fermentation in a large-scale reactor, using a continuous system, which is more advantageous than batch or fed-batch operations (uniform product quality, reductions in labor and cleaning costs, equipment size, and others) and gives high product yield in less time, increasing the process productivity [4]. The fermented broth was distilled and the volatile compounds present in the produced drink where identified.

MATERIALS AND METHODS

Cheese whey

Cheese whey (Quinta dos Ingleses, Caíde de Rei, Portugal) was centrifuged (2220 *g*, 20 min) to remove fines and cream, pasteurized at 65 ºC using a plate heat exchanger, and then applied to a 10000 Da ultrafiltration unit to concentrate the proteins. A protein rich fraction (the concentrate) and a low protein fraction (the permeate) were obtained. The permeate (lactose initial concentration of 50 g/L) was kept at 4 ºC in a holding tank before feeding to the 1000 L fermentation vessel.

Microorganism and cultivation conditions

The yeast strain *Kluyveromyces marxianus* ATCC 10022 was used in the experiment. The yeast was maintained at 4 ºC on slants containing a medium with the following composition (g/L): KH_2PO_4 (5), $(NH_4)_2SO_4$ (2), $MgSO_4.7H_2O$ (0.4), yeast extract (1), and lactose (20).

Inoculum and continuous fermentation

The inoculum was prepared by transferring the yeast strain to 100 mL sterile whey solution in 500 mL Erlenmeyer flasks. The culture was grown overnight in a rotary shaker, at 30 ºC and 120 rpm. In order to inoculate the 1000 L bioreactor, increasing volume inocula (1/5 to 1/10 of the volume of the inoculated vessel) were prepared in cheese whey till a 100 L volume inoculum was obtained.

Continuous experiments were performed in a 1000 L stainless steel air-lift bioreactor with 700 L working volume. The bioreactor was filled with pasteurized and deproteinized whey, inoculated with the 100 L of inocula and operated batch-wise until residual sugar concentration was negligible. Continuous operation was started by feeding fresh whey solution to the bottom of the reactor with a dilution rate of 0.2 h^{-1}. Whey solution was kept at 4 ºC to avoid any decomposition and was fed to the reactor under aseptic conditions using a centrifuge pump. During the experiment, the temperature was maintained at 30 ºC and the pH was controlled at 4.0 by automatic addition of H_3PO_4. The system was aerated with filtered air (0.1 vvm), and was kept operating for two months.

Distillation

Fermented broth distillation was carried out in a stainless steel pot still of 500 L. The fermented broth was transferred to the vessel up to 3/4 of its capacity in order to be distilled and the distilled product (alcoholic grade of approx. 50%) was poured into glass bottles.

Analytical Methods

Ethanol was quantified by high-performance liquid chromatography in a Jasco Chromatograph equipped with a refractive index (RI) detector and a Chrompack column (300 × 6.5 mm) at 80 ºC, using 0.005 mol/L H_2SO_4 as the eluent, a flow rate of 0.3 mL/min and sample volume of 20 µL.

Minor volatile constituents were determined by extraction with dichloromethane and subsequent analysis of the extracts by gas chromatography mass spectrometry (GC-MS) using a Varian 3400 chromatograph and an ion-trap mass spectrometer Varian Saturn II. Samples of 1 µL were injected in a capillary column coated with CP-Wax 52 CB (50 m x 0.25 mm i.d., 0.2 µm film thickness, Chrompack). The temperature of the injector was programmed from 20 °C to 250 ºC, at 180 ºC/min. The oven temperature was held at 60 ºC, for 5 min, then raised from 60 ºC to 250 ºC, at 3 ºC/min, held 20 min at 250 ºC and finally programmed to go from 250 ºC to 255 ºC at 1 ºC/min. Helium N60 at 103 kPa was used as carrier gas. The detector was set to electronic impact mode (70 eV), with an acquisition range from 29 m/z to 360 m/z, and an acquisition rate of 610 ms. Identification of volatiles was performed using the software Saturn version 5.2 (Varian). All the compounds were quantified as 4-nonanol equivalents.

Major volatile constituents were analyzed directly, without any previous treatment, on a Chrompack CP-9000 gas chromatograph equipped with a Split/Splitless injector and a flame ionization detector (FID). A capillary column, coated with CP-Wax 57 CB (50 m x 0.25 mm i.d., 0.2 µm film thickness, Chrompack), was used. The temperature of the injector and detector were both set to 250 ºC. The oven temperature was held at 60 ºC, for 5 min, then raised from 60 ºC to 220 ºC, at 3 ºC/min, and finally held 10 min at 220 ºC. Helium 55 at 103 kPa was used as carrier gas, and the split vent was set to 13 mL/min. Before injection of 1 µL in the splitless mode (for 15 s), the sample was added with 4-nonanol (internal standard) to reach a final concentration of 213.6 mg/L. Quantification of volatiles, as 4-nonanol equivalents, was performed with CP-Maître software version 2.5 (Chrompack), by comparing retention indices with those of pure standard compounds.

RESULTS AND DISCUSSION

The whey spirit produced after fermented broth distillation contained 35.4% v/v ethanol. Other forty volatile compounds were identified, among of which, higher alcohols were quantitatively the most abundant group, but several esters, acids, and terpenes were also present. The higher alcohols are normally the group with the highest concentration in distillates, given to them a flavoring aroma and an essential character [5]. The European legislation demands minimum requirements for these aromatic substances higher than 140 g/hL AA (absolute alcohol) [6]. In the spirit obtained in our study, the total concentration of higher alcohols was superior to this minimum requirement (equivalent to 540.85 g/hL AA), thus satisfying the European legislation.

Major volatile compounds in whey spirit

The concentration of major volatile compounds present in whey spirit is given in Table 1. Isoamyl alcohol, isobutanol, 1-propanol, and 2-methyl-1-butanol were the higher alcohols found in major quantity. Among the esters, ethyl acetate was the most abundant (138.2 mg/L), while among the aldehydes; acetaldehyde (36.7 mg/L) was found in major concentration. According to some authors [7–9], ethyl esters (mainly ethyl acetate), alcohols with three or more carbons, and acetaldehyde, are the major agents responsible for the flavor of alcoholic beverages and their amounts specify the quality of the distillate. Ethyl acetate, for example, has a significant effect on the organoleptic characteristics of distillates. This ester has a pleasant aroma of ‘‘fruity’’ properties, which turns vinegary at levels above 150 mg/L, adding spoilage ‘‘notes’’ to the beverage [9, 10]. The ethyl acetate concentration in whey spirit was in a level suitable to confer a pleasure flavor. Besides ethyl acetate, 1-propanol can also be a bacterial spoilage index, since its presence can be resulted of a possible microbial spoilage during storage under unfavorable conditions of the raw material before distillation [9]. The concentration of 1-propanol in whey spirit was similar to those reported for other distillates, such as tsipouro, grappa, whiskies and cider brandies [9], suggesting that the whey fermentation and storage were properly controlled. The concentration of 2-methyl-1-propanol in whey spirit can also be well compared to those found in these distilled beverages [9].

Table 1. Concentration of major volatile compounds present in the whey spirit by GC/FID.

		Concentration (mg/L)
Aldehydes	acetaldehyde	36.7
Esters	ethyl acetate	138.2
Alcohols	2-methyl-1-butanol (isopentyl alcohol) + 3-methyl-1-butanol (isoamyl alcohol)	175.5 + 886.6
	2-methyl-1-propanol (isobutyl alcohol)	542.1
	1-propanol	266.0
	2-butanol	34.7
	2-phenyl ethanol	7.8
Acids	acetic acid	79.9

Amyl alcohols (3-methyl-1-butanol and 2-methyl-1-butanol) are formed during fermentation by deamination and decarboxylation reactions from iso-leucine and leucine, respectively [11]. Such compounds constitute quantitatively the greater part of the higher alcohols in most of distilled beverages, being considered predictors of sensory character in the distilled product [6, 12]. In the whey spirit they were also the higher alcohols found in major quantity, with a total concentration of 1062.1 mg/L (Table 1), similar to those found in samples of other distillates, such as tsipouro, grappa, wine brandies, bourbon and malt whisky [9]. Increased concentration of amyl alcohols (having an aromatic description of ‘‘alcoholic’’, ‘‘sweet’’ and ‘‘choking’’) can contribute negatively to the aroma of the distillate [13]. Similarly, the 2-phenyl ethanol, which introduces a pleasant aroma to distillates, described as ‘‘rose-like’’, ‘‘sweet’’ and ‘‘perfume-like’’, just have a positive influence on the aroma of beverages when present at low concentrations [13]. In whey spirit, it was found in a concentration of 7.8 mg/L, equivalent to 2.20 g/hL AA. A close similar value (2.22 g/hL AA) was observed for samples of bagaceiras [12]; while in aguardiente samples [14], lower concentration values were detected (1.8 g/hL AA).

Low molecular mass carbonyls (aldehydes and ketones) are normally found in alcoholic beverages as by-products of yeast fermentation, intermediates in the formation of fusel oil and as a result of alcohol oxidation at various stages of beverage production. Nevertheless, their presence is not desirable because some of them are responsible for unpleasant organoleptic properties of alcoholic drinks [15]. In the present study, acetaldehyde was the only carbonyl identified among the major volatile compounds, but its concentration value (36.7 mg/L) was low when compared to other alcoholic distillates such as tsipouro or grappa [9]. This low concentration value is interesting because elevated acetaldehyde concentration gives a pungent irritating odor to the beverage, and can be health hazards [16].
In general, the major volatile compounds identified in whey spirit are similar to those present in other alcoholic beverages, like sake, mescal produced from *Agave salmiana*, or mouro. It should be pointed out that methanol was not found (by GC/FID) in whey spirit that is benefic since it has been reported a highly toxic effect for this compound (maximum legal limit 1000 g/hL of 100% vol. ethanol [17]), whose ingestion or inhalation can cause blindness or death [16]. Methanol is formed from pectin by pectolytic enzymes that hydrolyze the methoxyl group during fermentation [6, 9]. The whey spirit probably did not contain methanol because pectin does not exists in cheese whey.

Minor volatile compounds in whey spirit

Table 2 list the 30 compounds identified as minor components in whey spirit, and their concentrations. Despite the low concentration of minor compounds, their presence is relevant because they harmonically synergize to produce the characteristic beverage aroma [8].
An abundant group of ethyl esters was detected among the minor volatile components in whey drink. According to Mingorance-Cazorla et al. [10] the yeast used in the fermentation process has a great influence on ester production. The ethyl esters are compounds associated with the pleasant fruity flavors and flowery smell, and they have been detected in other alcoholic beverages such as tequila, mescal, mouro and others [6–8]. Besides the flavor characteristics, the ethyl lactate ester, particularly, is able to stabilize the distillate flavor and softens the harsh flavor characteristics when present at low concentrations [9], as found in whey drink. Three of the ethyl esters identified in whey spirit, namely, hexanoate, octanoate, and decanoate, are considered to have quantitatively a small participation compared to the other volatile compounds and determine a vastly profiled aromatic character for the spirits [5]. Otherwise, isoamyl acetate and phenyl-ethyl acetate, also found in whey drink, are mostly responsible for the flowery and fruity aroma of the distillates [5].
Several higher alcohols were also identified among the minor volatile components in whey drink, the 1-hexanol being the compound present in major quantity. This alcohol has a positive influence on the aroma of the distillate when occurs in concentrations up to 20 mg/L. Increased concentration of 1-hexanol, having an aromatic description of ‘‘coconut-like’’, ‘‘harsh’’ and ‘‘pungent’’, can contribute negatively to the product aroma. At even higher levels the organoleptic characteristics of the distillate are seriously impaired (‘‘green’’ flavor) [13]. The low 1-hexanol concentration found in whey spirit (≈ 0.6 mg/L) can be considered to affect positively the flavor of the product.
Besides ethyl esters and higher alcohols, the minor components in whey distillate also included some aldehydes, acids and terpenes. Among the aldehydes, furfural was identified in higher proportion. This compound is formed during distillation due to degradation of fermentable sugars (pentoses) caused by heating in acid conditions and/or Maillard reaction [18]. Thus, high amounts of furfural might be attributed to the presence of high quantities of residual pentose sugars, due to unfavorable fermentation conditions of the substrate [9]. Its odor is reminiscent of bitter almond and cinnamon and it has a toxic character. Therefore, it should be present in distillates only in low concentrations. Furfural concentration in whey spirit was very low (≈ 0.1 mg/L).
The acids identified among the minor volatile components in whey spirit included long chain fatty acids such as hexanoic, octanoic, nonanoic and dodecanoic acids, which are considered of low flavor effect to the distillates [6]. Table 2 shows their concentrations in the beverage obtained in this study. Such values were lower than those observed for other distilled drinks such as bagaceiras [12], aguardiente [14], and mouro [6].
Four terpenes were identified in whey based drink, including geranic acid, linalool, geraniol, and α-terpineol. The terpenes content is considered to be a positive quality factor to the beverage because they contribute to its aroma, serve to differentiate it from other beverages, and supply floral nuances to the drink [13]. In whey spirit, this compound was found in a concentration of 110.2 μg/L, a value well above of its perception threshold of 25 μg/L [19].

Table 2. Concentration of minor volatile compounds present in the whey spirit by GC/MS.

		Concentration (μg/L)
Aldehydes	furfural	101.1
	benzaldehyde	49.3
	1-heptanal	26.5
Esters	ethyl butanoate (ethyl butyrate)	1742.7
	ethyl hexanoate (ethyl caproate)	1417.1
	ethyl octanoate (ethyl caprylate)	1323.9
	phenyl-2-ethyl acetate	875.5
	3-methyl-butyl acetate (isoamyl acetate)	831.0
	ethyl 3-methyl butanoate	540.9
	ethyl 2-methyl butanoate	466.6
	ethyl decanoate (ethyl caprate)	301.6
	ethyl 2-hydroxypropanoate (ethyl lactate)	83.3
	ethyl 9-decenoate	76.2
	diethyl succinate (diethyl butanedioic acid)	42.4
Alcohols	1-hexanol	591.5
	1-butanol	351.6
	1-octanol	345.3
	1-heptanol	207.0
	3-methyl-1-pentanol	181.4
	2-heptanol	169.1
	3-methyl-3-buten-1-ol	88.5
Acids	octanoic acid (caprylic acid)	279.7
	hexanoic acid (caproic acid)	133.4
	3-methylbutanoic acid + 2-methylbutanoic acid	75.7
	dodecanoic acid (lauric acid)	57.2
	nonanoic acid (pelargonic acid)	26.9
Terpenes	geranic acid	171.2
	linalool	110.2
	geraniol	55.5
	α-terpineol	49.5

CONCLUSIONS

Forty volatile compounds were identified in the alcoholic drink produced by continuous fermentation of whey with *K. marxianus*. Most of them are similar to those reported for other alcoholic drinks, although the concentration values are different. Higher alcohols and ethyl esters were the most abundant compounds present, contributing thus for the greatest proportion of the total aroma. Some short and long chain fatty acid esters that contribute to fruity and flowery aroma were also present, and the volatile compounds that can be harmful to the health (methanol, acetaldehyde and ethyl acetate) were found at low levels. Considering that the quality of alcoholic drinks can be evaluated by the relation between isoamyl alcohol/2-methyl-1-propanol and 2-methyl-1-propanol/1-propanol, which have to be higher than unity, it was concluded that a novel spirit of acceptable organoleptic character can be produced by continuous fermentation of whey with *K. marxianus*.

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